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MAMMALIAN SODIUM CHANNEL PROTEINS

This invention relates to a novel voltage-gated sodium ion channel (herein "sodium channel"), nucleotides coding for it, vectors and host cells containing the same and methods of screening for modulators of said channel for the alleviation of pain and use in hypersensitivity pathologies.

Voltage-gated sodium channels are responsible for the rising phase of the action potential and as such, play a key role in mediating electrical activity in excitable tissues. The sodium channel is activated in response to depolarisation of the membrane. This causes a voltage-dependent conformational change in the channel from a resting, closed conformation to an active conformation, the result of which increases the membrane permeability to sodium ions (1,2).

Voltage-gated sodium channels comprise a multi-subunit complex consisting of a large (230-270kDa) highly glycosylated alpha (α) subunit which is usually associated with one or two of the smaller beta (b) subunits (b1 and b2) (3). The alpha subunits of voltage-gated sodium channels form a large multigene family which has expanded over recent years and at least nine different genes have now been identified in mammals (4-10). This alpha subunit consists of four homologous domains (DI-IV), each containing six potential α-helical transmembrane segments (SI-S6) which make-up the pore forming region. Domains critical for the function of the channel are highly conserved throughout the family of voltage-gated sodium channels. These include the S4 voltage sensors, the loop between domains III and IV which is involved in the inactivation of the channel and the SSI and SS2 segments of the extracellular loop between transmembrane regions S5 and S6, which are responsible for the channels vestibule and ion selectivity (11-13). b subunits appear to have a role in altering the kinetics of the sodium channel during activation and inactivation gating. Expression of the b subunits has been associated with an increase in peak current and a role in trafficking of the a subunit to the membrane (14 –17).

The most potent blocker of voltage gated sodium channels is the puffer fish toxin, tetrodotoxin, (TTX). While most voltage-gated sodium channels are inhibited by low nanomolar concentrations of TTX, there are two channels which are only inhibited by micromolar concentrations of TTX. These are the major cardiac channel (h1 or SKM2) and the sensory neurone specific channel (SNS/PN3) (3,6,7).

Sensory neurones of mammalian dorsal root ganglion (DRG) cells transmit sensory information from the periphery to the central nervous system and are known to express at least three distinct kinetic types of voltage-gated sodium currents. The small diameter neurones co-express a rapidly inactivating, fast TTX-sensitive current and a slowly activating and inactivating TTX-resistant sodium current. The larger diameter cells only express TTX-sensitive sodium currents which have intermediate activation and inactivation kinetics (19,20). This electrophysiological analysis has now been supported by molecular distribution studies, which suggest that there is a dynamic expression of voltage-gated sodium channels in DRG neurones which can change during development, response to injury and upon exposure to inflammatory mediators (21-24). The small diameter neurones are unmyelinated and are involved in the transmission of pain impulses, these are the so called c-fibres or nociceptive neurones (25).

Recent experimental evidence has associated and implicated sodium currents with the chronic pain and hypersensitivity pathologies of both inflammatory and neuropathic origin. For example in the small diameter nociceptive neurones, hyperalgesic agents such as prostaglandin E_2 (PGE_2) and serotonin enhance TTX resistant sodium currents and decrease the threshold for inactivation (26-28). Neuronal injury produces dramatic changes in sodium channel expression and distribution, for example accumulation of TTX-sensitive sodium channels at the neuroma of lesioned axons is thought to be responsible for formation of ectopic discharges (29, 30). In each case the neuronal hyperexcitability that results is highly likely to contribute to the induction and maintenance of this sensitised state. It follows that voltage-gated

sodium channels in sensory neurones may provide a highly tractable and attractive target for the development of novel analgesic and anti-hypersensitivity agents.

This supposition is supported by the observation that anaesthetic, anticonvulsant and antiarrythmic drugs, each with sodium channel blocking activity, can produce analgesia. For example, it has been recognised that sub-anaesthetic doses of lignocaine and bupivocaine elevate pain thresholds in man (31,32). In addition the anticonvulsant agents, phenytoin, carbamazepine and the class Ia antiarrythmic agent mexilitene are used clinically for neuropathic pain (33-35). The anticonvulsant lamotrigine is also weakly analgesic (36).

This invention provides a novel voltage-gated sodium channel specifically found in the small diameter subset of mammalian sensory neurones. This novel channel will be termed sensory neurone specific 2a (SNS_{2a}). The term "SNS_{2a}" as referred hereinafter will principally mean rat SNS_{2a} but may also be reference to the human form of the channel.

Nucleotide sequence analysis of SNS_{2a} rat cDNA (SEQ.I.D.NO:1) reveals a 5298bp open reading frame which encodes a 1765 amino acid protein (SEQ.I.D.NO:2). This deduced protein sequence shares many of the characteristic features associated with the voltage-gated sodium channel gene family, for example SNS_{2a} contains four homologous repeat domains each comprising six putative membrane spanning segments. A serine residue (S-355) is found at the site critical for TTX sensitivity and based on experiments with SNS/PN3, this residue should confer TTX resistance on clone SNS_{2a} (37). The predicted first intracellular loop region connecting the first and second repeat domains is considerably shorter than the corresponding region in many of the other voltage-gated sodium channels including SNS/PN3, the cardiac channel and the brain channels. Computer generated alignment of SNS_{2a} against the other members of the voltage-gated sodium channel gene family shows this ion channel to be distinct from any of the channels identified to date.

One aspect of the invention therefore provides an isolated mammalian sensory neurone sodium channel protein as set forth in SEQ.I.D.NO:2. Preferably the sodium channel of the invention is found in the neurones of the dorsal root ganglia. The sodium channel protein may be derived from any mammalian species, preferably the rat or human.

Included within the invention are variants of the sodium channel SNS_{2a}. Such variants include fragments, analogues, derivatives, and splice variants. The term "variant" refers to a protein or part of a protein which retains substantially the same biological function or activity as SNS_{2a}.

Fragments can include a part of SNS_{2a} which retains sufficient identity of the original protein to be effective for example in a screen. Such fragments may be probes such as the ones described hereinafter for the identification of the full length protein. Fragments may be fused to other amino acids or proteins (so-called "fusion" proteins) or may be comprised within a larger protein. Such a fragment may be comprised within a precursor protein designed for expression in a host. Therefore in one aspect the term fragment means a portion or portions of a fusion protein or polypeptide derived from SNS_{2a}.

Fragments also include portions of SNS_{2a} characterised by structural or functional attributes of the protein. These may have similar or improved chemical or biological activity or reduced side-effect activity. For example fragments may comprise an alpha helix or alpha -helix forming region, beta sheet and beta-sheet forming region, turn and turn forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, amphipathic regions (alpha or beta), flexible regions, surface-forming regions, substrate binding regions and regions of high antigenic index.

Fragments or portions may be used for producing the corresponding full length protein by peptide synthesis.

Derivatives include naturally occurring allelic variants. An allelic variant is an alternate form of a protein sequence which may have a substitution, deletion, insertion rearrangment or addition of one or more amino acids, which does not substantially alter the function of the protein. Derivatives can also be non-naturally occurring proteins or fragments in which a number of amino acids have been substituted, deleted or added. Proteins or fragments which have at least 70% identity to SNS_{2a} are encompassed within the invention. Preferably the identity is at least 80%, more preferably at least 90% and still more preferably at least or greater than 95% identity for example 97%, 98% or even 99% identity to SNS_{2a}.

Analogues include but are not limited to precursor proteins which can be activated by cleavage of the precursor portion to produce an active mature protein or a fusion with a compound such as polyethylene glycol or a leader/secretory sequence to aid purification.

A splice variant is a protein product of the same gene, generated by alternative splicing of mRNA, that contains additions or deletions within the coding region (Lewin N (1995) Genes V Oxford University Press, Oxford, England). The present invention provides splice variants of the SNS_{2a} sodium channel that occur naturally and which may play a role in changing the activation threshold of the sodium channel.

The protein or variant of the present invention may be a recombinant protein, a natural protein or a synthetic protein, preferably a recombinant protein.

A further aspect of the invention provides an isolated and/or purified nucleotide sequence e.g. DNA or RNA which encodes a mammalian sodium channel as described above, or a variant thereof. Also included within the invention are anti-sense nucleotides or complementary strands.

Preferably, the nucleotide sequence encodes a rat or human sodium channel. The nucleotide sequence may comprise the sequence of the coding portion of the nucleotide sequence shown in SEQ.I.D.NO:1.

A nucleotide sequence encoding a sodium channel of the present invention may be obtained from a cDNA or a genomic library derived from mammalian sensory neurones, preferably dorsal root ganglia.

The nucleotide sequence may be isolated from a mammalian cell (preferably a human cell), by screening with a probe derived from the rat or human sodium channel sequence, or by other methodologies known in the art such as polymerase chain reaction (PCR) for example on genomic DNA with appropriate oligonucleotide primers derived from or designed based on the rat or human sodium channel sequence and/or relatively conserved regions of known voltage-gated sodium channels. A bacterial artificial chromosome library (BAC) can be generated using rat or human DNA for the purposes of screening.

The nucleotide sequences of the present invention may be in form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the sodium channel or variant thereof may be identical to the coding sequence set forth in the SEQ.I.D NO:1, or may be a different coding sequence which as a result of the redundancy or degeneracy of the genetic code, encodes the same protein as the sequences set forth therein.

A nucleotide sequence which encodes an SNS_{2a} sodium channel may include: a coding sequence for the full length protein or any variant thereof: a coding sequence for the full length protein or any variant thereof and additional coding sequence such as a leader or secretory sequence or a proprotein sequence: a coding sequence for the full length protein or any variant thereof (and optionally additional coding sequence) and non-coding sequences, such as introns or non-coding sequences 5` and/or 3` of the coding sequence for the full length protein.

The invention also provides nucleotide variants, analogues, derivatives and fragments which encode SNS_{2a}. Nucleotides are included which preferably have at least 70% identity over their entire length to SNS_{2a}. More preferred are those sequences which have at least 80% identity over their entire length to SNS_{2a}. Even more preferred are polynucleotides which demonstrate at least 90% for example 95%, 97%, 98% or 99% identity over their entire length to SNS_{2a}.

The present invention also relates to nucleotide probes constructed from the nucleotide sequences of an SNS_{2a} sodium channel protein or variant thereof. Such probes could be utilised to screen a dorsal root ganglia cDNA or genomic library to isolate a nucleotide sequence encoding an SNS_{2a} sodium channel. The nucleotide probes can include portions of the nucleotide sequence of the SNS_{2a} sodium channel or variant thereof useful for hybridising with mRNA or DNA in assays to detect expression of the SNS_{2a} sodium channel or localise its presence on a chromosome using for example fluorescence *in situ* hybridisation (FISH) as described in the examples.

The nucleotide sequences of the invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the protein of the present invention such as hexa-histidine tag or a hemagglutinin (HA) tag or allows determination in screening assays of effective blockage of SNS_{2a} or its modulation.

Nucleotide molecules which hybridise to SNS_{2a}, or to complementary nucleotides thereto also form part of the invention. Hybridisation is preferably under stringent hybridisation conditions. One example of stringent hybridisation conditions which is sometimes used is where attempted hybridisation is carried out at a temperature of from about 35°C to about 65°C using a salt solution which is about 0.9 molar. However, the skilled person will be able to vary such conditions as appropriate in order to take into account variables such as probe length, base composition, type of ions present, etc.

The nucleotide sequences of the present invention may be employed for producing the SNS_{2a} sodium channel protein or variant thereof by recombinant techniques. Thus, for

example the nucleotide sequence may be included in any one of a variety of expression vehicles or cloning vehicles, in particular vectors or plasmids for expressing a protein. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences. Examples of suitable vectors include derivatives of bacterial plasmids: phage DNA: yeast plasmids; vectors derived from combinations of plasmids and phage DNA and viral DNA. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

More particularly, the present invention also provides recombinant constructs comprising one or more of the nucleotide sequences as described above. The constructs comprise an expression vector, such as a plasmid or viral vector into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises one or more regulatory sequences to direct mRNA synthesis, including, for example, a promoter, operably linked to the sequence. Suitable promoters include: CMV, LTR or SV40 promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector may contain an enhancer and a ribosome binding site for translation initiation and transcription terminator.

Large numbers of suitable vectors and promoters/enhancers, will be known to those of skill in the art, but any plasmid or vector, promoter/enhancer may be used as long as it is replicable and functional in the host.

Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts include mammalian expression vectors, insect expression vectors, yeast expression vectors, bacterial expression vectors and viral expression vectors and are described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY., (1989). A preferred vector is pBK-CMV.

The vector may also include appropriate sequences for selection and/or amplification of expression. For this the vector will comprise one or more phenotypic

selectable/amplifiable markers. Such markers are also well known to those skilled in the art.

In a further embodiment, the present invention provides host cells capable of expressing a nucleotide sequence of the invention. The host cells can be, for example, a higher eukaryotic cell, such as mammalian cell or a lower eukaryotic cell, such as a yeast cell or a prokaryotic cell such as a bacterial cell. Suitable prokaryotic hosts for transformation include E-coli. Suitable eukaryotic hosts include HEK293 cells.

Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

The SNS_{22} a sodium channel protein is recovered and purified from recombinant cell cultures by methods known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography and lectin chromatography. Protein refolding steps may be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The proteins and nucleotides sequences of the present invention are preferably provided in an isolated form. The term "isolated" means that the material is removed from its original environment (e.g., the naturally-occurring nucleotide sequence or protein present in a living animal is not isolated, but the same nucleotide sequence or protein, separated from some or all of the materials it co-exists with in the natural system, is isolated). Such nucleotide sequence could be part of a vector and/or such nucleotide sequence or protein could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. The proteins and nucleotides sequences of the present invention are also preferably provided in purified form, and preferably are purified to at least 50% purity, more preferably about 75% purity, most preferably 90% purity or greater such as 95%, 98% pure.

The present invention also provides antibodies specific for the SNS_{2a} sodium channel. The term antibody as used herein includes all immunoglobulins and fragments thereof which contain recognition sites for antigenic determinants of proteins of the present invention. The antibodies of the present invention may be polyclonal or preferably monoclonal, may be intact antibody molecules or fragments containing the active binding region of the antibody, e.g. Fab or $F(ab)_2$ The present invention also includes chimeric, single chain and humanised antibodies and fusions with non-immunoglobulin molecules. Various procedures known in the art may be used for the production of such antibodies and fragments.

The proteins, their variants especially fragments, derivatives, or analogues thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. Antibodies generated against the SNS_{2a} sodium channel can be obtained by direct injection of the polypeptide into an animal, preferably a non-human mammal. The antibody so obtained will then bind the protein itself. In this manner, even a sequence encoding only a fragment of the protein can then be used to generate antibodies binding the whole native protein. Such antibodies can then be used to locate the protein in tissue expressing that protein.

The antibodies of the present invention may also be of interest in purifying an SNS_{2a} protein and accordingly there is provided a method of purifying an SNS_{2a} or any portion thereof which method comprises the use of an antibody of the present invention.

The present invention also provides methods of identifying modulators of the sodium channel. Screens can be established for SNS_{2a} enabling large numbers of compounds to be studied. High throughput screens may be based on ^{14}C guanidine flux assays and fluorescence based assays as described in more detail below. Secondary screens may involve electrophysiological assays utilising patch clamp technology or two electrode voltage clamp to identify small molecules, antibodies, peptides, proteins, or other types or compounds that inhibit, block, or otherwise interact with the sodium channel. Tertiary screens may involve the study of the modulators in well characterised rat and

mouse models of pain. These models of pain include, but are not restricted to, intra-plantar injection of inflammatory agents such as carageenan, formalin and complete freunds adjuvant (CFA). Models of neuropathic pain such as loose ligature of the sciatic nerve are also included.

The invention therefore provides a method of assaying for a modulator comprising contacting a test compound with the sodium channel and detecting the activity or inactivity of the sodium channel. Preferably, the methods of identifying modulators or screening assays employ transformed host cells that express the sodium channel. Typically, such assays will detect changes in the activity of the sodium channel due to the test compound, thus identifying modulators of the sodium channel.

For example, host cells expressing the sodium channel can be employed in ion flux assays such as $^{22}Na+$ ion flux and ^{14}C guandinium ion assays, as described in the examples and in the art, as well as the SFBI fluorescent sodium incubator assays as described in Levi et al., (1994) J Cardiovascular Electrophysiology 5 -241-257 and voltage sensing dyes such as DiBAC. Host cells expressing the SNS_{2a} sodium channel can also be employed in binding assays such as the 3-H- batrachotoxin binding assay described in Sheldon et al., (1986) Molecular Pharmaeology 30:617-623; the 3-H- saxitoxin assay as described in Rogart et al (1983) Proc Natl. Acad. Sci. USA 80: 1106-1110; and the scorpion toxin assay described in West et al., (1992) Neuron 8: 59-70.

In general, a test compound is added to the assay and its effect on sodium flux is determined or the test compound's ability to competitively bind to the sodium channel is assessed. Test compounds having the desired effect on the sodium channel are then selected.

Modulators of the sodium channel will impede or prevent the transmission of impulses along sensory neurones and thereby be useful in the treatment of acute, chronic or neuropathic pain i.e. production of an analgesic effect and or in the treatment of hypersensitivity pathologies. Modulators of the present invention and

medicaments comprising the same may also be particularly useful in the the treatment of pain associated with inflammatory conditions e.g. arthritic conditions such as rheumatoid arthritis. The invention therefore provides a modulator of a protein or a variant thereof as described above identifiable by a method described above for use in therapy. The invention further provides the use of a modulator of a sodium channel protein optionally identifiable by a method described above for the manufacture of an analgesic or anti-hypersensitvity medicament. Such medicaments are typically pharmaceutical compositions comprising an effective amount of the modulator together with stabilisers, preservatives and the like as known and called for by accepted pharmaceutical practice. Effective amounts of the modulator, i.e. therapeutically effective amounts, may be determined according to routine methods known to those skilled in the art. The invention further provides a method of treatment which comprises administering to a patient an effective amount of a modulator of a protein as described above. Treatment protocol will depend on a number of considerations including the severity of the condition to be treated, such considerations being within the purview of the attending physician. Modulators of the present invention may also be used in conjunction with e.g. simultaneously, sequentially or separately with modulators of the ion channel disclosed in PCT/GB96/01523, the entire contents of which are incorporated herein by reference and to which the reader is specifically referred.

Complementary or anti-sense strands of the nucleotide sequences as hereinabove defined can be used in gene therapy. For example, the cDNA sequence or fragments thereof could be used in gene therapy strategies to down regulate the sodium channel. Antisense technology can be used to control gene expression through triple-helix formation of antisense DNA or RNA, both of which methods are based on binding of a nucleotide sequence to DNA or RNA.

A DNA oligonucleotide is designed to be complimentary to a region of the gene involved in transcription thereby preventing transcription and the product of the sodium channel. The antisense RNA olignucleotide hybridises to the mRNA in vivo

and blocks translation of the mRNA into the sodium channel. Antisense oligonucleotides or an antisense construct driven by a strong constitutive promoter expressed in the target sensory neurons would be delivered either peripherally or to the spinal cord, optionally by minimally invasive e.g. endoscopic, means.

The regulatory regions controlling expression of the sodium channel gene could be used in gene therapy to control expression of a therapeutic construct in cells expressing the sodium channel.

In accordance with further aspects of the present invention, there is provided an isolated sodium channel derivable from the dorsal root ganglion, particularly the nociceptive neurones of the dorsal root ganglion of a mammal, e.g. rat or human which has a IC50 for TTX of about 1μM.

Further provided is a protein which has a primary amino acid sequence with at least 90% or greater identity with the primary amino acid sequence of the protein of SEQ.I.D.NO.2.

Also provided is a recombinant polynucleotide comprising a sequence which comprises the sequences as defined in each of SEQ.I.D.NO:3 to 17 wherein ascending numerical order represents the order in which the SEQ.I.D. NO:3 to 17 is read in the 5' to 3' direction. The recombinant polynucleotide may further comprise sequences spaced before, between and after the sequences (in terms of reading direction) defined in SEQ.I.D.NO:3 to 17 such that when the polynucleotide is expressed in a host cell, e.g. mammalian cell, a functional sodium channel is encoded.

Brief description of the Figures:

Figure 1 is a summary of the rat SNS_{2a} ion channel fragments isolated, and probes used for analysis.

Figure 2 depicts the position of SEQ.I.D.NO:3 to SEQ.I.D.NO:17 relative to the rat SNS_{2a} clone.

Figure 3 shows the localisation of human SNS_{2a} to human chromosome 3p21.

Figure 4 shows rat multiple tissue Northern Blot probed with SNS_{2a}. Lane 1 = DRG: Lane 2 = Spinal cord: Lane 3 = Total brain: Lane 4 = Adrenal gland: Lane 5 = Heart: Lane 6 = PC12: Lane 7 = PC12 + NGF: Lane 8 = RNA markers.

Figure 5 In situ hybridisation in rat DRG tissue using an SNS_{2a} specific probe. Figure 5a) shows a sense probe and 5b) shows an anti-sense probe.

Figure 6 shows localisation of SNS_{2a} to human DRG

Figure 7 Northern blot probed with SNS_{2a} using DRG tissue taken from rat pain models. Lane 1 = Control DRG: Lane 2 = DRG + 24 hours complete freunds adjuvant (CFA): Lane 3 = DRG + 24 hours sciatic nerve cut: Lane 4 = DRG + 48 hours sciatic nerve cut: Lane 5 = DRG + 7 days sciatic nerve cut.

Figure 8 illustrates the three vectors into which rat SNS_{2a} has been cloned: pBluescript, pCI-neo and pCIN5.

Figure 9: shows photomicrographs of SNS_{2a} and SNS/PN3 staining for mRNA and protein. D.E. F confirm SNS_{2a} labelling is found exclusively in small neurons (10-25µm diameter). Double labelling for SNS_{2a} and SNS/PN3 mRNA and protein (G.H.I.J) shows colocalisation in small neurons (arrows): larger neurons can be seen positive for SNS/PN3 but negative for SNS_{2a} (arrowheads).

Figure 10: Immunoprecipitation Western blot showing specific staining in the lanes from cells transfected with SNS_{2a} DNA with the two antipeptide antibodies designed to SNS_{2a}. Control lanes. where cells were transfected with yellow fluorescent protein (YFP) show no staining.

Figure 11: shows biophysical and pharmacological properties of recombinant rat SNS_{2a} Na^+ channels expressed in HEK293T cells. Representative current records are shown along with their peak current-voltage relationships (A.B). Capacitance transients have been blanked for clarity. Pannel C illustrates the effect of TTX on SNS_{2a}. It is resistant to sub μM concentrations of TTX. compared with the nM sensitivity of TTX sensitive sodium channels.

The following examples are for illustrative purposes only and are not limiting of the invention.

Example 1: DRG cDNA Library screening

Example 1a: Obtaining The Probe

A sodium channel probe was generated to allow screening of a rat DRG cDNA library with the aim to identify novel sodium channels present in the DRG. A pan specific sodium channel probe was obtained from Polymerase chain reaction (PCR) experiments using rat genomic DNA as the template and degenerate PCR primers designed from within the 3' coding regions of the brain II. heart. skeletal muscle and glial voltage-gated sodium channel. The oligonucleotide primers used for this analysis were as follows. FORWARD PRIMER (5' CCTG/CGTCATGTTCATCTAC 3'. and REVERSE PRIMER (5' CTCATAA/GGAA/GAC/TCTTGGAG/AGGG 3'). The PCR conditions used. were 94°C for 30 seconds. 50°C for 1 minute and 72°C for 2 minutes. These conditions were used for 35 cycles of PCR. The resulting PCR products were separated on a 1% agarose gel and cloned into the TA cloning kit (Invitrogen) according to manufacturers instructions. The resulting clones were taken for sequence analysis and separate clones were identified with identical sequence to the published rat brain II. heart. skeletal muscle and glial voltage-gated sodium channels.

A rat DRG cDNA library was constructed in λZAP Express™ Bacteriophage system (Strategene), allowing it to be directionally cloned within the pBK-CMV excision vector. Briefly, lumbar DRG tissue was removed from adult rats and frozen in liquid nitrogen until ready for processing. Total RNA was extracted using RNAzol B (Biogenesis) according to the manufacturers instructions. This method is based on the guanidine isothiocyanante and phenol/chloroform extraction method developed by Chomczynski and Sacchi, Analytical Biochemistry (1987) **162**, 156-169. Poly (A+) RNA was then isolated from the total RNA pool by oligo dT celluloise chromatography (invitrogen) as per manufacturers instructions. 5μg of this poly (A+) rat DRG RNA was used as the starting template for cDNA library synthesis. This was carried out exactly as stated in the Stratagene Instruction manual for construction of a ZAP express cDNA library using the Gigapack III Gold cloning kit.

Initially two million plaque forming units from this library were screened (as outlined in DNA transfer and hybridisation and probing) with the pan specific sodium channel probe. The resulting positive plaques were purified to homogeneity (as outlined in the Strategene instruction manual for the construction of a ZAP express cDNA library using the Gigapack III Gold cloning kit) and subjected to sequence analysis. Several clones were obtained which demonstrated a novel sequence related to voltage-gated sodium channels. The longest of these clones has been annotated as LARI/QFL in figure 1. Figure 1 displays the key clones obtained from the DRG cDNA library screening. This novel sequence was a fragment of the sodium channel referred to in this invention as SNS_{2a}.

Subsequently, a further one and a half million plaques were screened using the probe (LARI/QFL), specific to this novel sodium channel. Further positive clones were obtained and verified by sequence analysis. The largest of these clones designated as clone 63.1 in figure 1 was 3.6 kb in length. Degenerate oligonucleotide primers were designed to perform RT-PCR reactions on DRG RNA. The primers used were as follows :

5' AGGGAGGTCACCGGCCTGAAA/C 3';

and 5'AGTGGATA/CGAGAA/CCATGTGGG 3'.

Conditions used were 94° C for 30 seconds. 50° C for 1 minute and 72° C for 2 minutes. These conditions were used for 35 cycles of PCR. The resulting PCR products were separated on a 1% agarose gel and cloned into the TA cloning kit (Invitrogen) according to manufacturers instructions. The resulting clones were taken for sequence analysis. This resulted in the discovery of the partial SNS_{2a} clone 18/14. This is annotated as 18/14 in figure 1 which illustrates the position of this clone relative to the full length sequence of SNS_{2a}. Two million plaques were screened in the third cDNA library screening using this probe designated as 18/14. (probe labelling as in hybridisation and probing). Analysis of the positive clones obtained from this screen resulted in the discovery of the fragments annotated in figure 1 as 16/24. 31/42 and the 3.4kb clone 71/72. The two clones designated 71/72 and 63.1 (figure 1) overlapped with each other thus allowing them to be joined together using a unique Bgl II (New England Biolabs) restriction site from position 2895 bp to 2900 bp of SNS_{2a}. This step generated the full length SNS_{2a} clone which is shown in SEQ.I.D.No.1.

SNS_{2a} has been assembled in the Sal1/NotI sites of the mammalian expression vector pBluescript (Stratagene). the SalI/NotI sites of the mammalian expression vector pCI-neo (Promega). SNS_{2a} was also cloned into the Clontech IRES vector pIRESneo. the multiple cloning site of this vector was modified to include NsiI/NotI sites which were used for cloning SNS_{2a} in the correct orientation. This allows for both transient and stable expression studies in mammalian cells such as HEK293 cells (ATCC).

Nucleotide sequence analysis of SEQ.I.D.NO:1 reveals a 5298bp open reading frame which encodes a 1765 amino acid protein (SEQ.I.D.no.2). This deduced protein sequence shares many of the characteristic features associated with the voltage-gated sodium channel gene family, however. the predicted first intracellular loop region connecting the first and second repeat domains is considerably shorter than the corresponding region in many of the other voltage-gated sodium channels including SNS/PN3. the cardiac channel and the brain channels.

Example 1b: DNA Transfer

The DNA was transferred onto a GeneScreen™ hybridisation transfer membrane (DUPONT) by placing on the surface of the phage infected plate for 1 minute. The membrane is washed with 1M NaOH twice for 2 minutes, followed by two neutralisation steps in 1M Tris (pH 7.4) for an additional 2 minutes. An additional duplicate lift was done with the filter on the plate for five minutes prior to the washing steps. The membrane is then air dried overnight or crosslinked using the UV Stratalinker (Stratagene).

Example 1c: Hybridisation and probing

The membranes were hybridised for 4 hours shaking at 60°C in a 10% dextran sulphate, 1% lauryl sulphate (SDS) (see solutions and media) and 1M NaCl solution. The probes used were LARI & QFL and 18/14 respectively, from the 5' and middle regions of 33b. The probe was labelled with [α ^{32P}] dCTP (Amersham) using the Rediprime™ DNA labelling system (Amersham), so as to obtain approximately 500,000 cpm of the labelled probe per ml of prehybridization solution. Briefly, 100ng of each probe was boiled for 3 minutes (denaturization) and then cooled on ice for 2 minutes in a total volume of 45µl. This was added to the labelling tube from the kit together with 3µl of 32P dCTP, followed by an incubation at 37°C for 30 minutes. 400µl of Herring Sperm DNA (Sigma) at a concentration of 400µg 50ml was added to the labelled probe and heated at 99°C for 3 minutes followed by rapid cooling on ice. The labelled probe was added and mixed well in the prehybridisation solution. The membranes were hybridised overnight at 55°C.

The membranes were then washed, first at room temperature, in 2x SSC (3M sodium chloride and 0.3M sodium citrate pH7) and 1% SDS (sodium dodecyl sulphate) for 5 minutes, followed by 2x SSC and 1% SDS for 30 mins at 50°C, and if necessary further washes with 1x SSC and 0.5% SDS or 0.1x SSC and 0.1% SDS for 30 mins at

the same temperature. The membranes were then exposed to Scientific Imaging Film AR (Kodak) using intensifying screens at -70°C overnight and the film developed.

Example 1d: Southern Blot analysis

PCR products which were separated using agarose gel electrophoresis were denatured in situ by shaking the gel slowly in 1.5M NaCl for 10 minutes followed by a 0.5M NaOH solution for 30 minutes. DNA transfer onto a GeneScreen™ hybridization transfer membrane (DUPONT) by capillary action occured overnight, followed by washing in 2x SSC for 2 minutes and left to air dry. The hybridization and probing was carried out in the same way as for the library screening.

Example 2: *In vivo* excision analysis

Approximately 6 phage plugs were removed from the agarose plate and placed in 500µl of SM buffer. Elution of the phage particles occured at room temperature while gently shaking for 2-3 hours. 1µl of ExAssist™ Helper phage (Stratagene) was added to 100µl of phage stock in SM buffer (see media and solutions) and incubated at 37°C for 15 minutes. 3ml of liquid broth (see media and solutions) was added, followed by shaking at 225rpm at 37°C for 3 hours. Heat shock at 70°C for 15 minutes was followed by centrifugation at 4000rpm for 15 minutes at 4°C. The supernatant was carefully decanted into sterile 50ml falcon tube and stored at 4°C until needed.

10µl and 100µl of the rescued recombinant plasmid (supernatant from the step above) was used to transform 200µl of XLOLR cells (Stratagene) at OD_{600} 1.0 and incubated at 37°C for 15 minutes. The samples are incubated for a further 45 minutes at 37°C

after the addition of 300µl of L-broth (see media and solutions), followed by spreading on kan-plates (15µg/ml) (see media and solutions) and incubation overnight at 37°C. Positive colonies were analysed by digest analysis using XhoI and EcoRI restriction enzymes followed by subsequent southern blot analysis.

Example 3: Transient expression of SNS_2 in mammalian cells

Mammalian cells such as HEK293 cells were plated 24 hours prior to transfection, such that they are 50-80% confluent for the transfection procedure. On the day of transfection fresh media was added to the cells. The transfection protocol to be used relies upon the calcium phosphate transfection method (CalPhos maximer, Clontech) although any transient transfection method can be used. Briefly, a solution referred to as solution A, was made up containing 2-4 µg of plasmid DNA per 4 x 10^5 cells, 5 - 30 µl of CalPhos maximer, 12.4 µl 2M calcium solution, sterile water to 100 µl. The following solution referred to as solution B was also made up comprising, 100 µl of HEPES buffered saline. Solution B was carefully vortexed while solution A was added dropwise. The mixed solutions were incubated at room temperature for 20 minutes. After this period the solution was gently vortexed and added to the cell culture medium. 200 µl of solution was used per 35 mm^2 vessel with 4 x 10^5 cells. The vessel was then gently rocked to distribute the solution. The cells were incubated at 37°C for 2-6 hours, after which the medium was removed by aspiration and the cells were washed with phosphate buffered saline. Fresh culture media was then added to the cells. Electrophysiological assays were carried out 24-72 hours post transfection or alternatively antibiotic selection was applied after 24 hours for the generation of stable cell lines.

Example 4: Northern blot analysis

20µg of total RNA from DRG, heart, spinal cord, adrenal glands, PC12 cells (ATCC), and PC12 cells pretreated with NGF were electrophoresed on a 1% agarose gel.

containing 8% formaldehyde. (The preparation of the total RNA was carried out as described in the construction of the rat DRG cDNA library). The gel was then blotted onto a Genescreen™ membrane as described previously in Example 1d and probed with the 18/14 probe as described in Example 1c. Exposure to Kodak X-AR film occurred overnight.

The results of this Northern blot analysis using the 18/14 probe, which was specific to SNS_{2a} demonstrated a transcript size of approximately 9kb in DRG cells, while no expression was observed in spinal cord, brain, adrenal gland, heart and the rat phenochromocytoma cell line (PC12) in the absence or presence of nerve growth factor (NGF) (figure 4). *In situ* hybridisation experiments performed on DRG sections demonstrated that SNS_{2a} expression was limited to the small diameter cells (figure 5). Similar *in situ* hybridisation experiments were performed on spinal cord and whole brain sections and no specific labelling was observed confirming the Northern analysis work.

The expression of SNS_{2a} in DRG tissue was studied in DRG tissue removed from two separate rat models of pain, namely the Complete Freunds Adjuvant (CFA) model and the sciatic nerve cut (axotomy) model. The expression of SNS_{2a} was studied by Northern blot analysis using the probe 18/14 as described earlier in this section. In the CFA model at the 24 hour time point, there was a significant increase in expression of SNS_{2a} however there was a significant decrease in the level of SNS_{2a} mRNA at the 48 hour and 7 day time periods in the axotomy model (figure 7). This important series of experiments demonstrates differential regulation of this novel channel SNS_{2a} in well characterised models of pain.

Example 5: Riboprobe generation

The 18/14 probe, which was specific to SNS_{2a} in northern blot analysis, was used to generate riboprobes. The 18/14 probe was cloned into the vector pCR-II (Invitrogen). Labelled RNA strands were transcribed *in vitro* from the vector promoters, SP6 and T7, using the DIG RNA labelling Kit (Boeringer Manheim). 1µg

linearised template DNA was used to produce 'run off' transcripts. DIG-UTP was used as a substrate and incorporated into the transcript. The amount of DIG labelled RNA generated was determined using the DIG Quantification and Control Teststrips (Boehringer Manheim). DIG labelled sense and anti-sense RNA probes were used for *in situ* hybridisation to stain rat DRG sections (see section on antibody generation).

Example 6: Antibody Generation

The octadecapeptide CNGDLSSLDVAKVKVHND relating to amino acid residues 1748 to 1765 of SNS_{2a} and the peptide EERYYPVIFPDERNC relating to amino acid residues 2 to 15 of SNS_{2a} were synthesised on a Biosearch 9500 peptide synthesiser using solid-phase Fmoc chemistry under conditions recommended by the suppliers. Cleaved peptide was purified by gel filtration and conjugated to purified protein derivative of tuberculin (PPD) using sulpho-SMCC. Dutch rabbits. presensitised against BCG. were immunised with the resulting conjugate emulsified in incomplete Freunds adjuvant. Rabbits were boosted at three week intervals and serum prepared from test bleeds 7 days after each injection. The specific antibody response was followed by indirect ELISA using free synthetic peptide as antigen. High titre antisera were used for further studies.

These anti-peptide antibodies directed to SNS_{2a} can be used in immunohistochemistry experiments. Several fusion protein antibodies have also been generated against SNS_{2a}. The PCR primers used to generate fusion peptides were as follows:

Fusion peptide 1 5' GATCGAATTCAAGGAGAAAATGTTTCAGGA 3' and 5' GATCGTCGACTCATTTGGTCTGCTCAAGGA 3'

Fusion peptide 2 5' GATCGAATTCGGCGGTGCCCTACCCACCTC 3' and 5' GATCGTCGACTCATTCCATTTCAACCCCTT 3'

Fusion peptide 3 5' GATCGAATTCAAGCACAACTGTGGCCCCAA 3' and 5' GATCGTCGACTCACATTATGAAGTCTTCGC 3'

The anti-peptide antibodies have been verified by specific staining of recombinant SNS_{2a} expressed in HEK293 cells (see section on transient expression of SNS_{2a}). HEK293 cells were transfected with a) SNS_{2a} DNA or b) YFP, yellow fluorescent protein, DNA as a control. 48 hours after transfection whole cell lysates, prepared with RIPA, were precleared using non-immune rabbit IgG and protein-A-sepharose followed by centrifugation. Immunoreactive proteins were then precipitated by adding specific antibodies to the resulting supernatants. Dissolved precipitates were subjected to SDS-PAGE and immunoblotted using a cocktail of anti-SNS_{2a} antibodies. Bound immunoglobulins were revealed using HRP-labelled secondary antibodies and ECL detection. Anti-SNS_{2a} antibodies showed specific binding to cells transfected with SNS_{2a} DNA, confirming the specificity of the antibodies and the presence of SNS_{2a} protein (see figure 10).

The anti-peptide antibodies and riboprobes have been used for *in situ* hybridization and immunohistochemistry to stain rat DRG sections. Fresh frozen L4 and L5 dorsal root ganglia from adult male Sprague Dawley rats were cut at 20μm, air dried and fixed, acetylated and hybridised with 500ng/ml digoxygenin-labelled riboprobes (see section on SNS_{2a} riboprobes). Sense probes were used as a control. The sections were photographed before incubating overnight in either SNS/PN3 or SNS_{2a} antibody (1:2000). Staining was achieved using a Vectastain elite ABC kit (Vector). For cell profile area analysis, sections stained for either SNS/PN3 or SNS_{2a} mRNA were randomly chosen, labelled cells with visible nuclei were drawn (SNS/PN3: n=212; SNS_{2a}: n=206) and areas calculated using NIH Image 1.61. Once again the antibody and riboprobe recognise the small diameter cell bodies of the peripheral sensory neurones (Figure 10, D and E). Profile area frequency distribution analysis of SNS_{2a}, compared to SNS/PN3, mRNA labelled cells demonstrates that expression of SNS_{2a} is restricted to small (15-30μm in diameter) cell bodies (figure 9, C and F).

This observation has been extended to human DRG tissue and this experiment demonstrates that the antibodies raised to the rat sequence do in fact cross react with the human SNS_{2a} channel.

Double labelling studies for mRNA and protein in the same section showed co-localization only in small diameter neuronal cell bodies (figure 9. G.H.I.J). Large neurons were frequently seen with a signal for SNS/PN3 mRNA or protein where SNS_{2a} protein or mRNA were absent. The co-localisation of SNS_{2a} with SNS/PN3 in small diameter cell bodies may be functionally significant.

Example 6: Antisense

Antisense oligonucleotides were synthesised to further validate the contribution of SNS_{2a} to the TTX-R current in DRG neurons. The antisense and mismatch oligos were designed to the 5` region of the SNS_{2a} nucleotide sequence from base pair 45 to 180, the sequences were as follows:

Antisense 1 5'-AGT ACC TCT CCT CCA TCT-3'
Mismatch 1 5'-AGT ACT CAT CCC TCA TCT-3'

Antisense 2 5'-CAC CGG GTA GTA CCT CTC-3'
Mismatch 2 5'-CAC GCG CTA GTC ACT CTC-3'

Antisense 3 5'-GTC TTT GGA CTT CTT CCT-3'
Mismatch 3 5'-GTC TGG TGA CTC TTT CCT-3'

The antisense oligonucleotides may be used *in vitro* with cultured rat DRG neurons to look at the effects of knockdown of SNS_{2a} protein on the electrophysiological properties of the DRG neurons. The behavioural effect of SNS_{2a} knockdown in rat DRG neurons maybe looked at *in vivo* using antisense delivery via cannulae into the spinal cord. Reduction of SNS2a protein levels maybe measured by quantitative western blots. A full electrophysiological analysis can then be carried out. This

permits analysis of the role of SNS_{2a} in normal nociception and in inflammatory and neuropathic pain models.

Example 7: Electrophysiology

Importantly, SNS_{2a} forms functional voltage-gated Na^+ channels when expressed in mammalian cells (e.g. HEK293T), as determined using whole-cell patch clamp electrophysiology.

Standard electrophysiological techniques were employed to measure whole-cell Na^+ currents (Hamill *et al.*. 1981). Voltage command protocols were generated and current records stored, via a Axopatch 200B amplifier, and a digidata1200 analog/digital interface (Axon Instruments) controlled by microcomputer (Viglen Pentium) using pCLAMP6 Clampex software (Axon Instruments). Signals were prefiltered at 5kHz bandwidth and sampled at 20kHz. Capacitance transients and series resistance errors were compensated for (80-85%) using the amplifier circuitry, and linear leakage currents were subtracted using an on-line 'P-4' procedure provided by the commercial software package. In most cases evoked Na^+ currents ranged from -600pA to -4500pA and thus the maximum estimated voltage drop across the compensated series resistance will amount to less than 4mV. Patch pipettes were fabricated from 1.5mm outside diameter borosilicate capillary glass (Clark Electromedical) using a micropipette puller (Sutter model P97), and fire polished (Narishige Microforge) to give final tip resistances of 2-4mΩ. A silver/silver chloride pellet was used as the bath reference electrode and the potential difference between this and the recording electrode was adjusted for zero current flow before seal formation. Cells were visualised using a Diaphot200 inverted microscope (Nikon) with modulation contrast optics at a final magnification of x400. High resistance seals (1-10GΩ) between pipette and neuronal cell membranes are achieved by gentle suction, and the 'whole cell' configuration attained by applying further suction.

For measurement of Na^+ currents in SNS_{2a} transfected HEK293 cells (see transfection methods) a bath solution containing (mM concentrations) NaCl 110, CaCl2 1, D-glucose 20, MgCl2 5, KCl 5 pH7.4 290-305mosm was used. The internal (pipette) solution contained CsF 120, NaCl 15, Cs-EGTA 10, HEPES 10, pH 7.25, 275-285mosm. Cells were held at a holding potential of –90mV and prepulsed to –140mV for 1s prior to depolarising steps. No active currents were observed in untransfected cells under these conditions.

In SNS_{2a} transfected cells membrane depolarisation evoked transient inward currents at potentials positive to –70mV that peaked at –20mV and reversed close to the Na^+ equilibrium potential (+57mV). The $V_{1/2}$ and slope parameters for the Boltzmann function describing Na^+ conductance were -45 ± 1mV and 5.3 ± 0.2mV/*e*-fold, respectively (n=5). Peak currents ranged from 0.2-0.8nA. When a single exponential function was fitted to the inactivation phase of the peak current a **t** value of 1.2 ± 0.1ms was obtained (n=5). SNS_{2a} currents were highly resistant to TTX; the estimated IC_{50} value was 1mM which is some 1000-fold less sensitive than recombinant brain Na^+ channels (Isom *et al.*, 1995). The key features of recombinant SNS_{2a} Na^+ channels expressed in HEK293T cells are summarised in Figure 11.

Multiple sequence alignment of SNS_{2a} with all of the voltage-gated sodium channels identified to date, suggests that SNS_{2a} may be the prototypical member of a new gene family, related to both TTX-R and TTX-S sodium channels. Consistent with this, SNS_{2a} displays a combination of TTX resistance (IC50 for TTX in the μM range) and channel biophysical properties, more characteristic of the TTX-S channels.

SNS_{2a} encoded a functional voltage-gated sodium channel when expressed in HEK293T cells, which displayed more rapid activation and inactivation kinetics and a lower threshold for activation than seen for SNS/PN3 (see PCT/GB96/01523) expressed in the same cells. Previous electrophysiological studies of small diameter DRG cells have provided data compatible with heterogeneity of the TTX-R current (Rizzo et al., 1994; Rush and Elliott, 1997; Scholz et al., 1998). We too have found that the TTX-R current profiles varied between cells: in a few cells the TTX-R current

biophysical properties were similar to the profile expressed by SNS_{2a} in HEK293T cells and in a small group of others. the kinetics were more similar to the profile expressed by SNS/PN3. Interestingly, we never observed TTX-R currents in DRG neurons that were activated at voltages as low as –60mV as was case for SNS_{2a}. This is consistent with our distribution data suggesting that SNS_{2a} is not found alone but is always expressed together with the higher threshold SNS/PN3 channel. In most cells, however, the current biophysical properties of TTX-R were intermediate between the two, suggesting that the current results from a combination of the activity of both SNS_{2a} and SNS/PN3 channels.

Example 8: Screening

Having established that SNS_{2a} has significant potential as a pain target a screening strategy has been determined in order to identify modulators of channel function. High throughput screens are based on assays such as ^{14}C guanidine flux assays and fluorescence based assays using both sodium indicator dyes such as SBFI and voltage sensing dyes such as DiBAC. Secondary screens involve electrophysiological assays utilising patch clamp technology or two electrode voltage clamp. Tertiary screens involve the study of modulators in rat and mouse models of pain.

The critical path depicting the key steps in the SNS_{2a} high throughput screen is shown below. The screen should aim to cover at least 200.000 compounds in the primary screen but may be as high as 1 million compounds. the hit compounds are then re-tested against mammalian cell lines expressing the brain and/or cardiac sodium channels. The tertiary screen will take compounds which are potent and selective and test them in a range of in-vivo pain models.

		Primary Screen		Secondary Screen		Tertiary Screen
>200K **Compounds**	→	**HTS:-**	→	**Selectivity/**	→	**In-vivo**

FLIPR/ G-Flux **Use Dependence**

Recombinant SNS_{2a} Cell Line

Brain/Cardiac (SKN-SH-SY5Y) N-Type Calcium

Isolated Nerve Inflammatory Pain Neuropathic Pain HT Patch Further Selectivity Detailed Electophysiology

The G-FLUX method is the method of choice and it has been further improved with the introduction of Cytostar-T plates (Amersham) which remove the necessity for digestion of the cells in triton and transfer into scintillation vials. Cytostar-T plates are standard format tissue culture treated plates in which the transparent base of each well is composed of polystyrene and scintillant that permits cultivation and observation of adherent cell monolayers. Radioisotopes brought in close proximity with the base by virtue of the biological process within the cells thereby result in the generation of light.

Guanidine Flux (G-Flux) assay

Mammalian cells stably over-expressing SNS_{2a} will be cultured in 96 well plates. One T225cm^3 flask will be sufficient for setting up ten 96 well plates with a volume of 100µl cell culture medium in each well. These plates are set up the night before each assay run. The culture medium is removed and 100µl of assay buffer (125mM Choline chloride. 50mM HEPES. 5.5mM Glucose. 0.8mM $MgSO_4$. 5mM KCl. pH 7.4 added. The test compounds are then added to the wells and pre-incubated for a period of 10 minutes. Scorpion toxin (0.31 mg ml^{-1}) and veratrine (1.25mg ml^{-1} (Sigma) will then be added to activate the sodium channel. these compounds hold the channel in a open conformation. The cells are incubated for a further 10 minutes prior

to the addition of ^{14}C guanidine (Amersham). This is incubated for a period of 3 minutes after which time the whole plate can be read on a scintilation counter.

Example 9: Cloning of human SNS_{2a}

The human SNS_{2a} gene has been cloned as a genomic DNA fragment. PCR experiments were performed on human genomic DNA, using oligonucleotide primers designed from the rat SNS_{2a} sequence. A fragment corresponding to the human SNS_{2a} gene was subsequently isolated and sequenced. A human bacterial artificial chromosome (BAC) library (Research Genetics) was then screened using PCR primers designed from human sequence. A 120kb BAC clone (BAC#4) was isolated which has been extensively characterised following the construction of a random library from the BAC clone. (see section below). This clone contains the gene encoding human SNS_{2a} (SEQ.I.D.NO: 3 to 17) shows regions where coding sequence has been obtained from the BAC clone against an idealised template.

This BAC clone (BAC#4) containing human SNS_{2a} was mapped to human chromosome 3p21 by fluorescence in situ hybridisation (FISH) (figure 3). The human SNS/PN3 gene has also been mapped to the same chromosomal locus. It is worthy of note that the human cardiac channel has also been mapped to chromosome 3p21. A new gene cluster of TTX-resistant sodium channels has therefore been identified on human chromosome 3.

Example 10: Purification of BAC DNA

BAC DNA was purified according to the Qiagen BAC DNA method. Briefly BAC liquid culture was inoculated into a 5ml starter culture of L broth with 12.5 μg/ml chloramphenicol selection. This was used to inoculate 200 ml L broth with (selection) which was then grown for 14 hours at 37 C with vigorous shaking. The culture was then centrifuged at 4500 x g for 20 minutes. The bacterial pellet was resuspended in 20 ml of buffer p1. 20 ml of P2 was added and the solution was mixed gently and incubated at 21 C for 5 minutes 20 ml of chilled buffer P3 was added.

solution mixed gently and incubated on ice for 15 minutes. Following centrifugation at 20000 x g for 30 minutes the supernatant was applied to an equilibrated Qiagen Tip 100. The column was washed twice with 10 ml of buffer QC. The DNA was eluted with five 1 ml aliquots of buffer QF, pre warmed to 65 C. The DNA was precipitated with 3.5 ml of isopropanol and centrifuged at 15000 x g for 15 minutes. The supernatant was removed and the pellet was washed with 2 ml of 70% ethanol and centrifuged at 1500 x g for 10 minutes. The pellet was finally air dried for 10 minutes and resuspended in water.

Example 11: Construction of Random Library from BAC Clone

This was an essential prerequisite to analyse the 120kb BAC clone containing the human SNS_{2a} gene.

5µg of BAC DNA in a volume of 50 µl was sonnicated in the cup horn, in two pulses of 1 second at power level 2, with cooling on ice for 1 minute between pulses. The overhanging or ragged ends, caused by the sonication, of the fragmented DNA molecules were made flush by the exonuclease or polymerase activity of T4 DNA polymerase. The components were as follows, 47.5 µl sonicated DNA, 20 µl 5 x T4 DNA buffer, 10ul 2mM each dNTP, 17.5 µl double distilled water, 5 ul T4 DNA pol (1 unit/µl Boehringer). This reaction mix was incubated at 37°C for 3 hours. The DNA was size selected with Pharmacia SizeSep 400 spin column. The resulting DNA fragments were ligated into a SmaI phosphatased pBluescript II SK vector (Stratagene) and subsequently transformed into XL1 blue competent E.coli (Stratagene). Individual colonies are PCR amplified with M13 reverse and M13-20 primers, which flank the insert. The PCR products were sequenced using the nested primers T3 and T7.

A second method was employed as above except the following T4 DNA polymerase repair, oligonucleotide linkers were ligated onto the DNA fragments. Using primers directed against sites within these oligos the DNA fragments were amplified by PCR. The lnker ligation reaction mix was set up as follows, 1 of sonicated BAC DNA, 5 µl

T4 DNA ligase (400 units / µl NEB). 5 µl 10 x ligase buffer, 2 µl linkers, 37.5 µl double distilled water, and incubated for 8 hours at 21 °C. PCR amplification was performed using 50 p.moles linker primers, 1 x buffer (Promega), 1.5mM $MgCl_2$, 200 µM each dNTP, Taq (Promega) 0.5 unit. The reaction volume was 50 µl and the PCR parameters: 94°C for 2 minutes, 94°C 30 seconds, 55°C for 1 minute, 72°C 2 minutes, for 40 cycles, 72°C 10 minutes. The resulting PCR products were ligated into the TA cloning vector (Invitrogen) and transformed in INVαF' competent E.coli (Invitrogen). The resulting PCR products were then sequenced with T3 and T7, which are nested primers.

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